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ORIGINAL PAPER

Extraction, purification and antioxidant activity of polysaccharides from bamboo leaves

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Abstract: Ultrasonic extraction (UE) was employed for the extraction of bamboo leaf polysaccharides (BLP). The influential parameters of UE procedure including extraction time, ultrasonic power and solid/liquid ratio were optimized by orthogonal experiments. DEAE-cellulose column chromatography was applied to purify BLP and then the radical scavenging activity of BLP was also evaluated. Optimal extraction conditions were: extraction time of 15 min, ultrasonic power of 300 W, and solid/liquid ratio of 1:15. Four kinds of polysaccharides were obtained by DEAE-cellulose column chromatography; the maximum superoxide radical scavenging rate (20.4%) of BLP was inferior to that of vitamin C (V_C, the control) and the hydroxyl radical scavenging rate (50%) was equivalent to that of V_C.

Keywords: bamboo leaves; polysaccharide; scavenging radical; separation and purification; ultrasonic extraction

Introduction

Bamboo, a perennial green plant, has its own sub-family, Bambusoideae, in the Gramineae family and is widely grown in the Asia-Pacific region, Eastern America and Western Africa (Jiang et al. 2004). Edible and medicinal values of bamboo leaves have been recorded for a long time (Huang et al. 2002). Recent studies showed that bamboo leaves contain a large number of bioactive polysaccharides and other active ingredients such as phenolic acids, flavones, and special amino acids (Lu et al. 2003; Yang et al. 2004).

Polysaccharides in bamboo leaf make unique health contributions to various functions of the human body such as immunity regulation, anti-oxidation, and tumor prevention (Zhang et al. 2009). Yet little information is available on chemical composition of bamboo leaves. The traditional extraction method for polysaccharide is hot-water treatment. This method is time-consuming and has a low extraction rate (Yao et al. 2009). Use of ultrasound might increase yield for extraction of proteins, medicinal compounds, and tea solids (Mason et al. 1996). Recently, ultrasound extraction (UE) has been increasingly used for extraction of polysaccharides due to its high efficiency, simplicity, high extraction rate, and complete components (Gao et al. 2009). During ultrasonic cavitation, mechanical and thermal effects can break cell tissues and improve the leaching rate of polysaccharides (Li et al. 2003; Hu et al. 2007). However, UE can change the structure of polysaccharides, thereby degrading the quality of the extracted compounds. The changes in structure and resulting degradation of polysaccharides are determined by extraction methods. Therefore, a comprehensive understanding of the effect of UE on polysaccharides is needed (Yang et al. 2008).

The objective of this study was to identify the best method for extraction of bamboo leaf polysaccharides (BLP) using UE. We also determined the hydroxyl and superoxide radical scavenging activity of BLP by measuring the antioxidant activity in purification and *in vitro*.

Materials and methods

Materials and reagents

Bamboo leaves were collected from *Bambusa rutila* planted in the bamboo garden of Shaanxi Normal University (China), dried at 60°C, powdered and then stored in bottles. The standard glucose solution was prepared in our laboratory. Diethylaminoethyl cellulose (DEAE-cellulose 52) was purchased from Beijing Ding States Biological Technology Co. Ltd. All other analytical grade chemicals were obtained from Xi'an Reagent Company (Xi'an, China).

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Extraction

The powdered bamboo leaves (5 g) were immersed in distilled water at increasing solid/liquid ratios (1:10, 1:15, 1:20 and 1:25). The extraction process was performed using an ultrasonic cleaner set at increasing time periods (5, 10, 15, 20 and 25 min), and at increasing power levels (150, 200, 250 and 300 W). The extract was filtered through a Whatman Nr1 filter paper and the filtrate was then concentrated with a rotary evaporator at 60°C under reduced pressure. The proteins in the extract were removed using the Sevag reagent. After removal of the Sevag reagent, the concentrate was mixed with four times its volume of 95% ethanol before the mixture was maintained overnight at 4°C to precipitate polysaccharides. The precipitate was collected by centrifuging at 3000 r/min for 10 min and then washed with acetone and ethanol to obtain crude polysaccharides.

Determination of polysaccharide

The polysaccharide solution was diluted to appropriate concentration and 1 mL of diluted polysaccharide solution was removed. Distilled water was added to reach a final volume of 2 mL. This was mixed with 1 mL phenol test solution and, after 10 min, 5-mL concentrated sulfuric acid. The mixture was shaken and left to stand for 20 min. The concentration of bamboo leaf polysaccharides (BLP) was determined by UV-spectrophotometry at 490 nm (Zhang et al. 2003). The concentration of polysaccharide solution was calculated according to the regression equation with glucose concentration X and absorbance A as: A = 13.476X - 0.0074 (correlation coefficient r = 0.999).

$$ERP\,(\%) = (C_1 \times V_1)/W_1 \times 100\,\%$$

where *ERP* is the extraction rate of polysaccharide, C_1 theconcentration of polysaccharide, V_1 the volume of polysaccharide solution, and W_1 is the dry weight of sample of bamboo leaves.

Optimization of extraction conditions

On the basis of a single-factor test for polysaccharide, an orthogonal test was set up according to the L_9 (3^4) orthogonal table. The extraction rate of polysaccharides was used as an indicator. Meanwhile, solid/liquid ratio, ultrasonic power and extraction time were considered as three factors. Each factor was tested in triplicate.

Table 1. Orthogonal factor level of bamboo leaf polysaccharides

Level	Factors		
	Extraction time (min)	Ultrasonic power (W)	Solid/liquid ratio (w/v)
1	10	200	1:15
2	15	250	1:20
3	20	300	1:25

Purification

To remove fine particles DEAE-cellulose was firstly soaked in water until it was fully expanded. The expanded DEAE-cellulose was soaked in NaOH (0.5 mol/L) solution for 1 h and the pH was adjusted to 9 by addition of distilled water. The mixture was then neutralized by HCl (0.5 mol/L) for 1 h. As a result, the DEAE-cellulose that was treated by 0.5 mol/L NaOH for 1 h was transformed to OH-type. A column (1 × 20 cm) was filled with DEAE-cellulose to the required density (without air bubbles) and the 2–3 cm gap at the top of the column was covered with water. After the pre-processing of DEAE-cellulose in the column, the sample was douched using distilled water at text concentrations of 0.05, 0.1, 0.3, 0.5 mol/L NaCl. The eluent flow rate was controlled at 1 mL/min and collected automatically with 3 mL in each test tube. Then, each sample was characterized by its absorption peak. The sulfuric acid-phenol method was used as a supplementary detection method (Ye et al. 2008).

Radical scavenging assay

Superoxide radical system assay
Superoxide radical-scavenging activity was estimated at 25°C by spectrophotometric monitoring of the inhibition of pyrgallol auto-oxidation (Marklund et al. 1974) with some modification. Solutions of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL of the BLP and V_C were prepared and placed at room temperature for further use. A 4.5-mL Tris-HCl buffer (0.05 mol/L, pH=8.2) was preheated at 25°C for 20 min and added to test tubes containing 1-mL samples previously dissolved in 0.4 mL pyrogallol solution (25 mmol/L), mixed thoroughly then incubated at 25°C for 4 min. The reaction was stopped by addition of 1.0 mL HCl (8 mol/L). The absorbance at 299 nm was determined to calculate the superoxide radical scavenging effect. Tris-HCl buffer was used as reference solution and distilled water was used as the blank. All determinations were carried out in triplicate.

Hydroxyl radical scavenging assay
Hydroxyl radicals were generated by iron-catalyzed Fenton-Haber-Weiss reaction (Jia et al. 1996; Guo et al. 2004). Solutions of 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL of BLP and V_C were prepared for future use. The mixture containing the following final concentration of reagents: 0.5 mL of salicylic acid (10 mmol/L), 3 mL of phosphate buffer (0.2 mol/L, pH=7.4), 0.5 mL of Fe^{2+}-EDTA (1:1, 3.8 mmol), 1 mL solution of sample, and 1 mL of H_2O_2 (4 mmol), reacted at 25°C for 90 min. The reaction was terminated after adding 1 mL of HCl (6 mol/L). NaCl (0.5 g) and 4 mL cold ether were fully mixed to the above reaction system; after standing the upper ether (about 3mL) was transferred to 10-mL centrifuge tube and dried at 40°C in water bath, and then 0.15 mL 10% trichloroacetic acid (W/V), 0.25 mL 10% sodium tungstate (W/V), and 0.25 mL 0.5% $NaNO_2$ (W/V) were added to the centrifuge tube to let set for 5 min; finally, 0.25 mL 1 mol/L KOH and distilled water were put into the tube, making the final volume reach to 4 mL. Absorbance was measured at 510 nm to estimate the scavenging effect. In the blank, the sam-

ple was substituted with distilled water. All determinations were carried out in triplicate. To determine superoxide radical and hydroxyl radical, radical scavenging effect (RSE) was calculated as:

$$RSE\ (\%) = (A_0 - A_1)/A_0 \times 100\%$$

where A_1 is the absorbance of the sample, and A_0 is the absorbance of the blank (1 mL deionized water instead of sample).

Results and discussion

Single factor experiment

Single factor analysis showed that the maximum extraction rate of polysaccharide was obtained at 250 W ultrasonic power (Fig. 1) and 15 min extraction time (Fig. 2). The extraction rate showed a single peak curve for both ultrasonic power and extraction time. The extraction rate of polysaccharides increased with increasing solid/liquid ratios (Fig. 3), peaking at a solid/liquid ratio of 1:20.

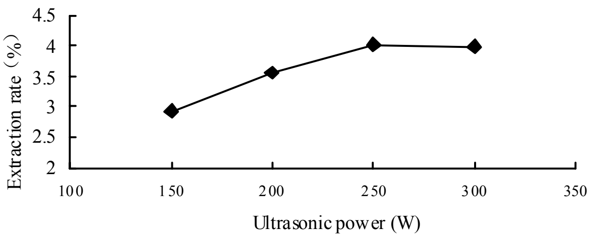

Fig. 1 Relationship between ultrasonic power and extraction rate of bamboo leaf polysaccharides

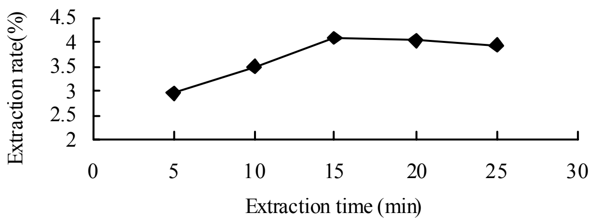

Fig. 2 Relationship between extraction time and extraction rate of bamboo leaf polysaccharides

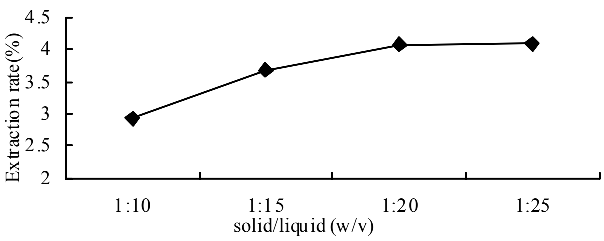

Fig. 3 Relationship between solid/liquid and extraction rate of bamboo leaf polysaccharides

Performance of orthogonal design

Based on the single-factor test above, the orthogonal table of L_9 (3^4) was selected to determine the optimum extraction condition in the orthogonal test, which was based on three levels: solid/liquid ratio, ultrasonic extraction time, and ultrasonic power. The results of orthogonal design and variance analysis showed that the factor most influencing the yield of BLP was extraction time, followed by ultrasonic power, and solid/liquid ratio (Tables 2 and 3). In summary, the optimal extraction conditions were ultrasonic time of 15 min, ultrasonic power of 300 W and solid/liquid ratio of 1:15.

Table 2. Results of orthogonal test of bamboo leaf polysaccharides using the UE

Test number	A Time (min)	B Power (W)	C Solid/liquid rate (w/v)	Empty columns	Extraction rate (%)
1	1（10）	1（200）	1（1:15）	1	2.43
2	1	2（250）	2（1:20）	2	2.51
3	1	3（300）	3（1:25）	3	3.01
4	2（15）	1	2	3	3.56
5	2	2	3	1	3.78
6	2	3	1	2	4.18
7	3（20）	1	3	2	2.98
8	3	2	1	3	3.11
9	3	3	2	1	3.67
K_1	7.95	8.97	9.72	9.88	
K_2	11.52	9.4	9.74	9.67	
K_3	9.76	10.86	9.77	9.68	
$\overline{K}_1$	2.65	2.99	3.24	3.29333	T=29.23
$\overline{K}_2$	3.84	3.13333	3.24667	3.22333	
$\overline{K}_3$	3.2533	3.62	3.25667	3.22667	
R	1.19	0.63	0.0167	0.07	

Table 3 Variance analysis of orthogonal test of bamboo leaf polysaccharides

Source of variation	sum of squares	degree of freedom	mean square	f value	significant level
A	2.12429	2	1.06214	227.0618	0.00438
B	0.65429	2	0.32714	69.93587	0.0141
C	0.00042	2	0.00021	0.04513	0.95682
D	0.00936	2	0.00468		
Errore	0.0094	2	0.00468		
Summation	2.78836				

Purification

The UV absorption spectra of BLP are shown in Fig. 4. The maximum absorption wavelength of BLP was at 205 nm.

The extraction solution was removed of protein and micromolecule impurities and bleached. It was then a mixture of multiple components of polysaccharides with differences in chemical composition, degree of polymerization, and molecular shape. To

get a single polysaccharide, the extraction solution needed to be purified. Hence, DEAE-cellulose was selected to analyze the component of BLP using the sulfuric acid-phenol method where absorption peaks were used for determination of polysaccharide. The results are shown in Fig. 5.

Four kinds of BLP eluted by distilled water, 0.1 mol/L NaCl, 0.3 mol/L NaCl, and 0.5 mol/L NaCl, respectively, were detected in the test (Fig. 5). The elution peaks emerged once in the above four elutions, respectively, while an elution peak did not emerge when using 0.5 mol/L NaCl.

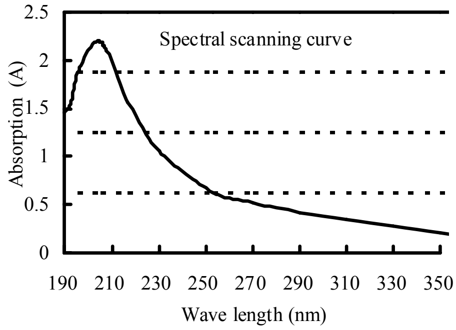

Fig. 4 UV absorption spectra of bamboo leaf polysaccharides

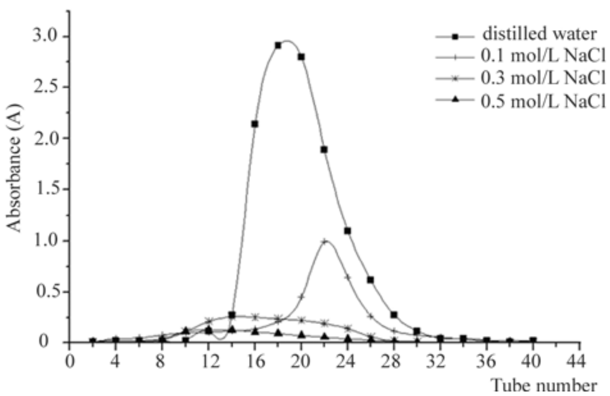

Fig.5 Chart of DEAE-cellulose column chromatography of bamboo leaf polysaccharides

Radical system scavenging assay

Superoxide radical scavenging assay

Superoxide radicals are very harmful to cellular components as a precursor of more active oxidative species such as singlet oxygen and hydroxyl radicals (Kanatt et al. 2007). Moreover, the superoxide radicals play significant role in the peroxidation of lipids, thus it is necessary to study the superoxide radical-scavenging effect of BLP. As shown in Fig. 6, BLP had a scavenging effect on superoxide anions. The scavenging rate increased slowly with increases in BLP concentration. The maximum scavenging rate was 20.4% and the value of IC_{50} could not be reviewed. In contrast, the scavenging activity of Vc was 85.81% at a dose of 1.0 mg/mL. Its IC_{50} value was 0.265 mg/mL according to the regression equation. This indicated that the scavenging effect of BLP was weaker than that of V_C.

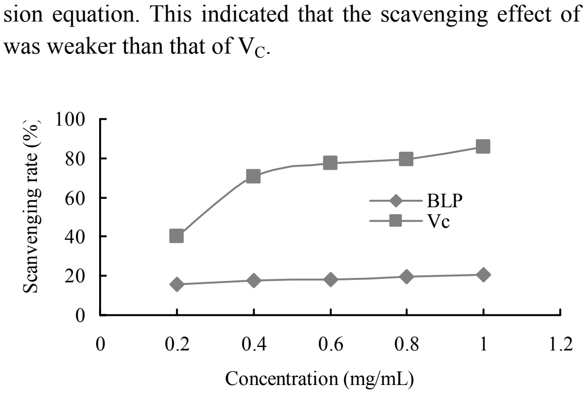

Fig. 6 Superoxide radical scavenging rate of bamboo leaf polysaccharides (BLP) and vitamin C (Vc).

Hydroxyl radical scavenging assay

The hydroxyl radicals, as the most reactive free-radical, are mainly responsible for the oxidative injury of biomolecules such as proteins, DNA, polyunsaturated fatty acids and nucleic acids. The hydroxyl radicals could be generated by reaction of Fe^{2+}-EDTA complex with H_2O_2 in the presence of a phosphate buffer. Salicylic acid attacked hydroxyl radicals to form a colored substance (2, 3-dihydroxybenzoic acid), then the yield of the colored substance was used to determine the amount of hydroxyl radicals. Added hydroxyl radical scavengers compete with salicylic acid for the hydroxyl radicals and diminish the colored formation (Fan et al. 2009). Fig. 7 shows an obvious scavenging effect on hydroxyl radicals between the BLP and VC in a concentration-dependent manner. The scavenging effect of BLP on hydroxyl radicals was equivalent to that of V_C. The IC_{50} values calculated for BLP and Vc from regression equations were 0.456 and 0.4 mg/mL, respectively.

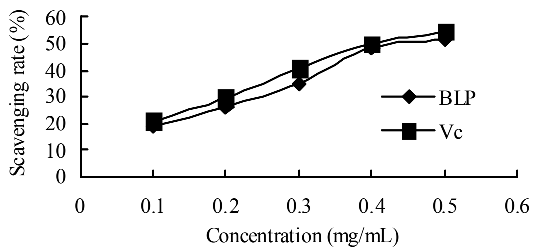

Fig. 7 Hydroxyl radical scavenging rate of bamboo leaf polysaccharides (BLP) and vitamin C (Vc)

Discussion

For the reaction dynamics of solid/liquid extraction, the extraction effect of plant tissue was mainly determined by the penetration speed of the active substance from the inside of cells to the surface. It was particularly important to undermine the walls of plant cells, so that the active substance had full access to the solvent. Owing to the effects of cavitation, grinding, stirring and

other special effects, the ultrasound can disrupt plant cells to enhance both the solvent penetration into the plant cells and the intracellular product release (Dong et al. 2003; Chen et al. 2007). Therefore, the ultrasonic extraction method, as a complementary means of solvent extraction (Matina et al. 1997), was useful for extraction of BLP.

Free radicals are the product of normal human metabolites. The number of free radicals in the body is so large that it would damage the biofilm, cell structure, and impede the body's normal metabolic activity and speed up the process of human aging (Ren et al. 1997; Chen et al. 2004). The present study shows that BLP is capable of scavenging superoxide and hydroxyl radicals; the scavenging capacity increased with increasing concentrations. However, the scavenging effect did not change when the polysaccharide concentration exceeded a threshold level.

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